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Novel method to characterize CYP21A2 in Florida patients with congenital adrenal hyperplasia and commercially available cell lines

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ABSTRACT

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder and affects approximately 1 in 15,000 births in the United States. CAH is one of the disorders included on the Newborn Screening (NBS) Recommended Uniform Screening Panel. The commonly used immunological NBS test is associated with a high false positive rate and there is interest in developing second-tier assays to increase screening specificity. Approximately 90% of the classic forms of CAH, salt-wasting and simple virilizing, are due to mutations in the CYP21A2 gene. These include single nucleotide changes, insertions, deletions, as well as chimeric genes involving CYP21A2 and its highly homologous pseudogene CYP21A1P. A novel loci-specific PCR approach was developed to individually amplify the CYP21A2 gene, the nearby CYP21A1P pseudogene, as well as any 30 kb deletion and gene conversion mutations, if present, as single separate amplicons. Using commercially available CAH positive specimens and 14 families with an affected CAH proband, the single long-range amplicon approach demonstrated higher specificity as compared to previously published methods.
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1. Introduction

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders of adrenal steroid biosynthesis. In most populations, nearly 90% of CAH cases are due to mutations in 21-hydroxlase (21OH), a key enzyme required for the conversion of progesterone and 17-hydroxyprogesterone (17OHP) to deoxycorticosterone and 11-deoxycortisol, respectively, in the cortisol and aldosterone biosynthesis pathways [1–3]. The clinical disease severity of CAH 21OH deficiency (21OHD) is dependent upon the levels of 21OH enzyme activity. Classic CAH can be defined as either salt wasting (SW) or simple virilizing (SV) [4]. In SW-CAH, there is complete loss of 21OH activity which results in the concurrent loss of cortisol and aldosterone synthesis, causing an elevated stress response and loss of electrolyte homeostasis in affected individuals. The absence of 21OH activity can lead to an adrenal crisis and cardiac arrest in an untreated individual. In SV-CAH, there is sufficient partial 21-OH activity (1–2%) to retain normal sodium balance while excess 17OHP is shunted into the androgen synthetic pathway, resulting in masculinization of external female genitalia and early onset of puberty for both sexes. In non-classical (NC) CAH, up to 50% of enzyme activity is retained and individuals present with milder late-onset phenotypes [2].

The gene encoding 21OH, CYP21A2, and a non-functional pseudogene, CYP21A1P, are located in the HLA class III region of chromosome 6. The CYP21A2 gene and the corresponding pseudogene each consist of 10 exons and are highly homologous, sharing approximately 98% nucleotide sequence identity [5]. The chromosomal region containing CYP21A2 and CYP21A1P is part of a 30 kb tandem duplication of four genes (serine/threonine Kinase RP, Complement C4, 21-hydroxylase CYP21 and Tenascin TNX) known as the RCCX module. The high degree of similarity between the gene repeats has been shown to result in unequal non-homologous recombination and gene conversion events between CYP21A2 and CYP21A1P [6–8]. Greater than 95% of inactivating CYP21A2 mutations associated with both SW- and SV-CAH are due to the transfer of CYP21A1P pseudogene sequence segments to the CYP21A2 functional gene through short intragenic conversion events, while large-scale conversions and the commonly occurring 30 kb-gene deletion alleles result from recombination between the CYP21A2 and CYP21A1P loci [9].

All newborns in the United States are tested for CAH as part of the recommended universal screening panel of conditions for newborn screening (NBS). The primary goals of NBS programs are to identify cases of CAH that might lead to early death due to an unrecognized salt wasting crisis as well as undetected cases of severe virilization that might result in erroneous sex assignments in females. The primary method for CAH detection is a fluoroimmunoassay assay that measures 17OHP from dried blood spot (DBS) specimens on filter paper card taken between 24 and 48 h of life. This method, however, is known to generate a great number of false-positive results due to a lack of antibody specificity, cross-reactivity with other steroid compounds in the DBS matrix, and elevated 17OHP levels in stressed, premature or sick newborns [2]. In order to reduce the overall number of false-positive results, states have implemented strategies to reduce the false positive rate for CAH screening. The first is the use of second-tier liquid chromatography tandem mass spectrophotometry (LC-MS/MS) methods to compare steroid profile measurements in the DBS specimen to increase testing accuracy [10–13]. However, LC-MS/MS second-tier assay has not yet been widely adapted by state screening programs. A recent study in Minnesota documented that while second-tier steroid profile assay reduced the rate of repeat testing of individuals due to equivocal test values, and improved the overall false positive screening rates, there was still a significant false negative rate and concern of the efficacy of the LC-MS/MS method. This result may be due to the use of the same DBS specimen utilized for the primary immune-assay and not a repeat DBS sample which would have increasing levels of 17-OHP for a true positive that accumulate with age in CAH [14,15]. The false positive rate for CAH screening has also been reduced by the collection and testing of a second DBS in some state newborn screening programs. Currently, nine states have a mandated second screen DBS collected from every infant at 8–14 days of age. Two states, Colorado and Texas, have published retrospective analyses of infants diagnosed with CAH on the basis of if they were detected with the single 17-OHP screen collected at 24–48 h or with the second DBS collected at 8–14 days. In both studies, the second DBS collected at 8–14 days identified cases of salt-wasting CAH that would have been missed with just a single screen (add Chan 2013, Therrell, 1998). While this second specimen increased the sensitivity of CAH screening, there is concern that the results of the second test may not be available prior to the onset of salt-wasting crises in affected infants [16].

An alternative second-tier test to enhance the specificity of CAH screening is molecular analysis of the CYP21A2 gene [17–19]. At birth, 17-OHP levels are normally elevated, especially in stressed infants. These high levels of 17-OHP in the DBS collected at 24–48 h affects not only the primary CAH immuno-assay results but also the steroid ratio measurements in the second-tier LC-MS/MS methods which are performed on the initial DBS. An advantage of a molecular second-tier screen for CAH is that mutation analysis of CYP21A2 would be independent of the 17-OHP levels and other cross-reactive confounding factors in the DBS. For molecular analysis to be practical for CAH NBS, the method must have the specificity required to target only the CYP21A2 gene without detecting related CYP21A1P pseudogene sequences and be able to detect large chromosomal rearrangements that lead to 21OHD. CYP21A2 genotyping methods from CAH samples have been described that use either oligo-ligation reactions, allele specific primer extension or reverse-hybridization assays [17,18,20–24]. These methods include a primary PCR reaction which is then used as a template for targeted genotyping assays or in the case of the reverse-hybridization assay, the PCR products are directly assayed on mutation-specific probes immobilized on test strips. Other methods based on hybrid capture enrichment of sheared DNA for next generation DNA sequencing have been described [25]. Complications of these methods include the requirement for multiple PCR reactions for each individual, and the inability to detect all recombinant alleles. Furthermore, these methods do not have sufficient specificity for the CYP21A2 or CYP21A1P genes for both forward and reverse PCR reactions or for oligonucleotide capture [26]. The purpose of this study was to develop a novel locus-specific PCR approach to individually amplify the CYP21A2 gene, the nearby CYP21A1P pseudogene, as well as any 30 kb deletion and large-scale conversion mutations, which could in the future be adapted by screening laboratories for CYP21A2 mutation detection.

2. Materials and methods

2.1. DNA samples

Genomic DNA was extracted from whole blood samples using the Gentra PureGene procedure from 60 non-affected CAH donors obtained from the Emory University Transfusion Medicine Program—CDC Specimen Management Bank Blood Services Program (Atlanta, GA). Genomic DNA from CAH affected samples or families with a CAH affected proband were purchased from the Coriell Institute for Medical Research and the European Collection of Cell Cultures (ECACC), including four CAH cases, one complete trio family and one mother–proband pair. An additional 14 families with an affected proband were recruited in collaboration with the University of South Florida (USF), comprising eight complete trio families and six mother–proband pairs. This collection was approved by the University of South Florida's (USF) Human Research Protection Program (HRPP) human subject research institutional review board. The USF samples were received as whole blood and DNA was extracted from a 5 ml aliquot with the QIAmp DNA Blood Maxi kit (Qiagen, Valencia, CA), and the remaining 5 ml of blood was spotted on 903 Whatman filter paper for future use for method development, and potential proficiency testing and quality control reference materials, similar to the procedure for cystic fibrosis DBS reference materials [27]. The DNA extraction from DBS was performed on three 3 mm punches per sample using the QIAmp DNA Micro kit (Qiagen). Extractions were performed according to the manufacturer's directions.

Table 1
PCR amplification primers.

Primer	Sequence	Region
CYP21A2-F	CTTGCTTCTTGATGGGTGAT(pt)C[a]	CYP21A2 forward
Tena36f2	AGGCGCTCGCTATGAGGTGAC	CYP21A2 reverse
ME0059	TCCCCAATCCTTACTTTTTGTC	CYP21A1P forward
TNXA-R	GGACACAGAAACTCCAGGTGGGAGT	CYP21A1P reverse
A2-Ex3F	CTTGGGAGACTACTCCCTGC	CYP21A2 exon 3, forward
A1P-Ex3F	ACCTGTCGTTGGTCTCTGCTC	CYP21A1P exon 3, forward
bPCR3-b76R	CCTCAGCTGCATCTCCACGATGTGA	CYP21A2 exon 6, reverse

Tena36f2 is from Lee and Lee (2003) and bPCR3-b76R is from Day and Speiser (1995).

[a] (pt) indicates phosphorothioate modification.

2.2. Locus-specific PCR amplification, gene sequencing and gene copy number

The CAH assay consists of four PCR amplifications performed with four sets of locus-specific primers to amplify the CYP21A2 functional gene (primers: CYP21A2-F, Tena36F2), the CYP21A1P pseudogene (primers: ME0059, TNXA-R), the CYP21A1P/CYP21A2 30 kb deletions (primers: ME0059, Tena36F2), and the CYP21A2/CYP21A1P large conversions (primers: CYP21A2-F, TNXA-R); two part amplifications are based on exon 3 and exon 6 sequences (A2-Ex3F, A1P-Ex3, bPCR3-b76R) (Table 1).

The PCR amplifications span the entire gene region from the promoters for both CYP21A2 and CYP21A1P to the downstream TNXB and TNXA genes for CYP21A2 and CYP21A1P, respectively (Fig. 1). An internal positive amplification control of AVPR2, located on the X chromosome (primers ME0004, ME0027) was duplexed in the reactions [21]. The expected sizes of the PCR products are as follows: CYP21A2—5.6 kb, CYP21A1P—6.1 kb, 30 kb deletion (CYP21A1P/CYP21A2 gene chimera)—6.2 kb, large-scale conversion (CYP21A2/CYP21A1P gene chimera)—5.5 kb and AVPR2—1.1 kb. Reference sequences for nucleotide variation for CYP21A2 are based on GenBank accession NM_000500.6 and protein sequences are based on GenBank accession NP_000491.2. PCR amplification was performed on a Gene Amp 9700 thermal cycler (Life Technologies). PCR reactions were performed in 25 μl total volume containing 25 ng of genomic DNA extracted from either whole blood or from DBS, 2X EmeraldAmp MAX HS PCR Master Mix (Takara, Otsu, Shiga, Japan), 0.2 μM of each test primer, 0.032 μM of control primers with the following touch-down cycling program: 98 °C for 3 min; 22 cycles of 98 °C for 10 s, 68 °C for 30 s (reduced 1 °C every 2 cycles), and 72 °C for 6 min followed by 25 cycles of 98 °C for 10 s, 57 °C for 30 s, and 72 °C for 6 min with a final 72 °C extension for 10 min and a hold at 4 °C. Products were visualized on a 0.9% agarose gel stained with ethidium bromide. Each PCR product was prepared for DNA sequencing with 5 μl of Exo SAP-IT (Affymetrix, Santa Clara, CA) and incubated at 37 °C for 30 min and heat-inactivated at 80 °C for 20 min. Products were diluted 1:50 in molecular biology grade water for Sanger cycle sequencing using 1:8 dilutions of BigDye Terminator V1.1 (Life Technologies) and sequencing primers as previously described (Table 1) [21]. Cycle sequencing products were purified using BigDye Xterminator and run on the 3730 DNA Analyzer (Life Technologies). Gene copy number analysis was performed by multiple ligation dependent probe amplification (MLPA) with the SALSA P050B2 Congenital Adrenal Hyperplasia kit following manufacturer's instructions and fragments were separated on the 3730xl DNA Analyzer (MRC-Holland, Amsterdam).

2.3. Bioinformatic analysis

Cycle sequencing data files were analyzed with SeqScape software from Life Technologies and MLPA fragments were analyzed with Coffalyser software (version 8; MRC-Holland). The novel intron 6 mutation c.738 + 41T > C was analyzed by *in silico* analysis with The Human Splicing Finder Tool (HSF, http://www.umd.be/HSF/) [28].
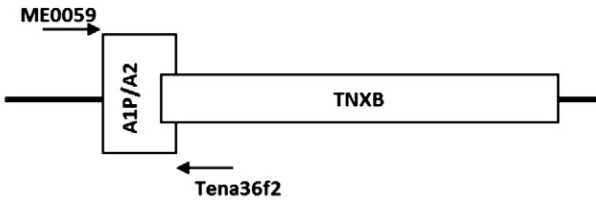
3. Results

A long-range locus-specific PCR approach was developed to specifically amplify CYP21A2, CYP21A1P, and 30 kb deletion or large scale CYP21A2 conversion alleles. PCR primers were designed for locus specificity based on nucleotide sequence variation between the promoter regions for CYP21A2 and the CYP21A1P pseudogene and on the divergence of the TNX-B and TNX-A gene sequences located downstream of CYP21A2 and CYP21A1P genes, respectively (Fig. 1).

The CYP21A2 gene amplification results in a 5.6 kb PCR fragment and the CYP21A1P pseudogene results in a 6.2 kb fragment. The forward primers were modified from Keen-Kim *et al.* so that the CYP21A2-specific sequence variation is located at the primer terminus [21]. Additionally, a phosphoro-thioate link was incorporated prior to the final 3′ base for the CYP21A2-F primer to inhibit the 3′-5′ exonuclease proof reading activity present in long-range EmeraldAmp polymerase [29]. The specificity of the CYP21A2 and CYP21A1P reverse primers was achieved by taking advantage of an approximately 150 bp deletion located in TNX-A exon 10 relative to the TNX-B gene. The CYP21A1P-specific TNXA-R primer spans the deletion breakpoint present within TNX-A while the CYP21A2-specific primer is located in the TNX-B region that is deleted in TNX-A [22].
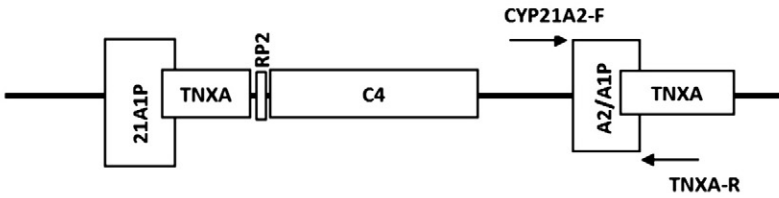
A. Common Chromosomal Arrangement and PCR Strategy
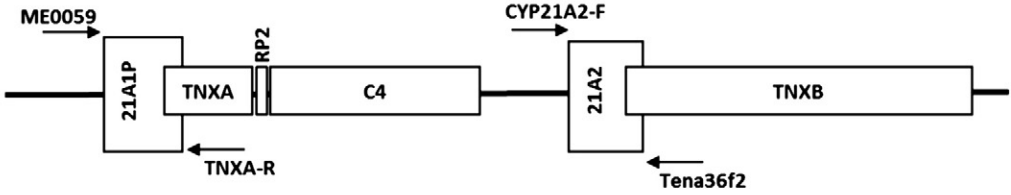
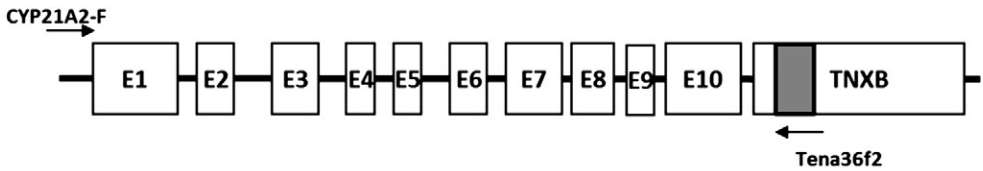### 30Kb Deletion – CYP21A1P/CYP21A2 fusion

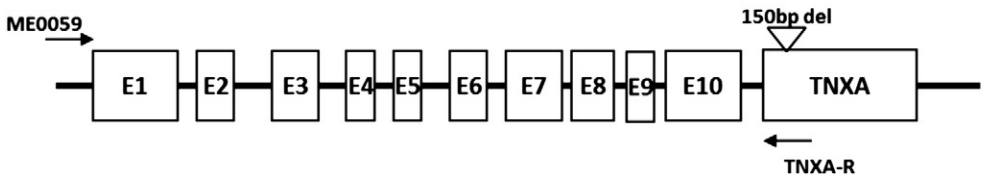### Gene Conversion – CYP21A2/CYP21A1P fusion

B. Locus-Specific Amplification

CYP21A2

CYP21A1P

Chromosomal rearrangements between CYP21A2 and the pseudogene, CYP21A1P, result in the common 30 kb deletion and large scale gene CYP21A2 conversion alleles. The 30 kb deletion allele, a chimeric fusion of the proximal region of the CYP21A1P pseudogene with the distal part of CYP21A2, is detected with the combination of the CYP21A1P forward primer with the CYP21A2 reverse primer that results in a 6.1 kb PCR fragment. Conversely, the large-scale conversion is a combination of the proximal region of CYP21A2 with the distal part of the CYP21A1P pseudogene and is detected with the combination of the CYP21A2 forward primer with the CYP21A1P reverse primer as a 5.5 kb PCR fragment (Fig. 1).

Each of the long-range PCR amplifications presented in this study was evaluated for specificity for the CYP21A2 gene and recombination alleles. In addition, the method presented here was compared with two previously published CYP21A2 amplification methods on 60 anonymous normal population controls and 9 commercially available CAH specimens. The first comparison was with a single PCR-amplification method developed by Keene-Kim, *et al.* where the locus specificity was based on a CYP21A2 vs. CYP21A1P gene reference sequence variation in the promoter and exon 10 3′ UTR [21]. The second method developed by Day *et al.* utilized an overlapping, two PCR-amplicon approach whose specificity was based on the Exon 3 8 bp deletion (c.332-339del) and Exon 6 cluster mutations (p.Ile236Asn, p.Val236Glu, and p.Met.239Lys) [20]. The exon 3 and exon 6 mutation sequences are present in the pseudogene, based on comparisons of nucleotide variation between CYP21A2 and CYP21A1P, and result in 21OHD when mutated in CYP21A2.

For each of the three methods, PCR amplifications for CYP21A2, CYP21A1P, the 30 kb deletion allele (CYP21A1P/CYP21A2) and large scale conversions (CYP21A2/CYP21A1P) were performed. For the normal population controls, our single long-range PCR method amplified the CYP21A2 in 60/60 specimens, CYP21A1P in 59/60, and resulted in no PCR amplification for the 30 kb or large conversion events. The Keene-Kim primer sets apparently amplified CYP21A2 in 58/60, CYP21A1P in 59/60, the 30 kb deletion in 2/60 and large conversions in 13/60. These included two specimens that tested positive for the CYP21A1P and conversions only, one positive for CYP21A2, CYP21A1P and the 30 kb deletion and one specimen positive for all four. The overlapping Day method amplification profile was consistent with our single long-range PCR results. For the Coriell and ECACC CAH-positive specimens, the three methods agreed for the CYP21A2, CYP21A1P and 30 kb deletion amplifications; however, the Keene-Kim primer sets were also positive for an apparent large conversion in two samples (Table 2).

The amplification products for the three different PCR methods were analyzed by DNA sequencing to resolve the discrepancies seen in the normal population controls and the commercially available CAH-positive samples. In each instance where the amplification profiles differed between the single long-range PCR amplification strategy presented here and the previously published Keene-Kim single amplicon method, the differences were due to areas of heterozygosity at the ME0066/ME0067 reverse primer site in the CYP21A2 and CYP21A1P exon 10 3′ UTRs. The absence of a CYP21A2 PCR fragment in two specimens, the presence of the two apparent 30 kb deletion alleles and the 13 conversion alleles were all false positive results. The reverse primers were designed to a region of the 3′ UTR sequence that contains two single nucleotide polymorphisms (c.*440C > T and c.*443T > C; rs6457475 and rs6457476, respectively) separated by two bases that are conserved between the functional and pseudogene reference sequence (CYP21A2-**C**GC**T**, CYP21A1P-**T**GC**C**). For samples that were negative for CYP21A2 amplification, the reverse primer site was homozygous for CYP21A1P-like sequence (TGCC) in CYP21A2, while the samples that were presumptive positive for the conversion alleles were heterozygous for these bases (CGCT/TGCC) in CYP21A2. In the sample that was positive for the CYP21A2, CYP21A1P and the 30 kb deletion allele, the region was heterozygous for these two bases in CYP21A1P and the sample that was positive for all four amplicons was heterozygous for both bases in both CYP21A2 and CYP21A1P. Thus, the

Fig. 1. CYP21A2 and CYP21A1P chromosome arrangement and PCR approach. PCR amplification approach for the chromosome 6 region containing CYP21A1P and CYP21A2 to detect the CYP21A2 locus and the common 30 kb deletion and large gene conversion alleles. A. The CYP21A1P pseudogene and functional CYP21A2 gene are part of a direct 30 kb chromosomal duplication in the HLA class III region of chromosome 6. Only a portion of the full repeat unit containing the RP-C4-CYP21-TNX gene families is shown for clarity. Recombination between the pseudogene and the CYP21A2 genes results in a deletion of the intervening TNXA-RP2-C4 genes and creates a fusion of the 5′ end of the pseudogene with the 3′ portion of CYP21A2 to form a CYP21A1P/CYP21A2 chimera. The 30 kb deletion is PCR amplified using a pseudogene specific forward primer and CYP21A2 gene reverse primer. For the large-scale gene conversion, there is a unidirectional transfer of pseudogene sequence to CYP21A2, resulting in a chimeric fusion of the 5′ end of CYP21A2 with the 3′ end of CYP21A1P. B. Promoter primers defined for CYP21A2 and CYP21A1P are paired with reverse primers designed to the downstream TNXB and A genes, respectively, for locus-specific amplification.

Table 2
Specificity and sensitivity of CYP21A2 PCR amplification procedures.

Assay	CYP21A2	CYP21A1P	30 kb deletion	Gene conversion
Normal population controls—N = 60				
Long-range	60	59	–	–
Keen-Kim	58	59	2	13
Day and Speiser	60	59	–	–
Commercially available CAH—N = 9				
Long-range	7	7	5	–
Keen-Kim	7	7	5	2
Day and Speiser	7	7	5	–
Florida CAH probands—N = 14				
Long-range	12	13	5	1
Day and Speiser	12	13	7	–

DNA sequence results indicate that in the control samples and commercially available positive samples tested, the DNA variation between CYP21A2 and CYP21A1P at the ME0066/ME0067 primers lacks locus specificity for CYP21A2. This method was not tested further in this study.

The single long range PCR amplification method presented here and the Day method were also used to characterize known commercially available CAH-positive samples and the 14 families from Florida with an affected CAH proband (Table 3). These two approaches produced concordant results with the commercially available samples. However, they produced different results in Florida families 4 and 8 due to the presence of an Exon 3 8 bp deletion allele in the CYP21A2 gene. This resulted in a positive 30 kb deletion allele amplification with the Day two amplicon approach but not with the single long-range amplicon. Similarly, Florida family 9 was positive for a long-range conversion with the single amplicon but negative by the Day approach due to a complex conversion tract that spans exon 1 to exon 2. This study shows that the placement of the locus specific primer in the two amplicon approach is limited to detecting recombination events only when they occur downstream of exon 3. These results were confirmed with MLPA copy number analysis of the CAH positive samples. The MLPA probes are designed to hybridize to those regions that differ between CYP21A1P and CYP21A2 such that the probes for CYP21A2 are equivalent to the functional alleles of sites mutated in CAH, p.Gly110Δ8 nt, p.Ile172Asn, exon 6 cluster and p.Gln318X in exon 3, exon 4, exon 6 and exon 8, respectively. For the CYP21A2 probes, a specimen carrying a mutation at one of these sites will be detected as a deletion in CYP21A2. Due to the allele-specific nature of the MLPA probes, any deletions or duplications detected in CYP21A2 or CYP21A1P should also corroborated with genotyping data [30,31]. The MLPA data for these specimens confirmed the DNA sequencing results from our long-range single amplicon approach.

The CYP21A2 and CYP21A1P genes were characterized by whole gene sequencing of the long-range single PCR amplicons in the normal controls, the commercially available CAH samples and the 14 Florida families with an affected CAH proband. In the normal controls, there were a total of 103 nucleotide locations that were polymorphic in either the CYP21A2 gene or the CYP21A1P pseudogene (Supplemental Table 1). Mutation analysis of the six commercial samples reported positive for CAH identified mutations in 10/12 chromosomes (Table 3). Five of these mutations were deletion alleles, four of which were detected by the 30 kb deletion PCR assay and the 5th deletion allele was an intragenic deletion of the promoter region through exon 8 detected by apparent hemizygous sequence for CYP21A2. These deletions were confirmed by MLPA copy number analysis. The breakpoints for the 30 kb deletions were identified as intron 3 (c.332-339del ^p.Ile172Asn; CH1), intron 7 (p.Gln318X^p.Arg356Trp; CH-3) and intron 6 (p.Leu307fx^p.Gln318X; CH-5) and have been previously described [8]. The remaining alleles identified were part of the 12 most common CYP21A2 mutations previously identified [9,32] : IVS2-13 A/C- > G (c.293-13A/C > G), p.Ile172Asn and p.Pro453Ser. No mutations were detected for sample DD1196 (ECACC) which has the phenotype listed as "mild." For the 14 Florida families with the affected CAH probands, there were 27 identified CYP21A2 mutated alleles. Chromosomal rearrangements account for 29% of the mutations which include seven 30 kb deletions and one conversion. The recombination breakpoints for the 30 kb deletion chromosomes were identified through DNA sequence comparisons: three in intron 3 (p.Gly110

Table 3
CYP21A2 mutations from commercially available sources and affected CAH probands.

Sample	Source	PCR profile				Mutation 1[c]	Mutation 2	Phenotype
		A2	A1P	30 kb Del	GC			
NA14732 (M)[a]	Coriell	+	+	−	−	Del.; promoter to exon8	No mutation detected	Normal
NA14733 (F)[a]	Coriell	+	+	+	−	30 kb Del. (CH-5)[b]	No mutation detected	Normal
NA14734 (P)[a]	Coriell	−	+	+	−	Del.; promoter to exon 8	30 kb Del. (CH-5)[b]	SW
DD1463 (P)[a]	ECACC	−	−	+	−	30 kb Del. (CH-1)[b]	30 kb Del. (CH-7)[b]	SW
DD1464 (M)[a]	ECACC	+	−	+	−	30 kb Del. (CH-1)[b]	No mutation detected	Normal
NA11781	Coriell	+	+	−	−	c.293-13A/C > G (IVS2-13 A/C- > G)	c.293-13A/C > G (IVS2-13 A/C- > G)	SV/SW
NA12217	Coriell	+	+	+	−	p.Ile172Asn	30 kb Del (CH-3)[b]	SV
DD0449	ECACC	+	+	−	−	c.293-13A/C > G (IVS2-13 A/C- > G)	p.Ile172Asn/p.Pro453Ser	SV
DD1196	ECACC	+	+	−	−	No mutation detected	No mutation detected	"Mild"
CAHAP1	USF	+	+	−	−	c.293-13A/C > G (IVS2-13 A/C- > G)	p.Val281Leu	SW/SV
CAHAP2	USF	+	+	−	−	c.293-13A/C > G (IVS2-13 A/C- > G)	p.Ile172Asn	SW/SV
CAHAP3	USF	+	+	−	−	p.Val281Leu	Promoter; c.1-126c > T, c.1-110T > A	NC
CAHAP4	USF	+	+	−	−	c.293-13A/C > G (IVS2-13 A/C- > G)	c.332-339del	SW
CAHAP5	USF	+	+	+	−	c.293-13A/C > G (IVS2-13 A/C- > G)	30 kb Del. (CH-1)[b]	SW
CAHAP6	USF	−	+	+	−	30 kb Del. (CH-1)[b]	30 kb Del. (CH-1)[b]	SW
CAHAP7	USF	+	+	+	−	c.293-13A/C > G (IVS2-13 A/C- > G)	30 kb Del. (Exon 7; p.Val281LeuL^p.Phe307fx)	SW
CAHAP8	USF	+	+	−	−	c.293-13A/C > G (IVS2-13 A/C- > G)	p.Gly110Δ8 nt	SW
CAHAP9	USF	+	−	+	+	30 kb Del. (CH-7)[b]	Conversion: p.Pro30Leu - IVS2-13 A/C- > G	SW
CAHAP10	USF	+	+	−	−	c.293-13A/C > G (IVS2-13 A/C- > G)	p.Ile172Asn	SW/SV
CAHAP11	USF	+	+	−	−	c.293-13A/C > G (IVS2-13 A/C- > G)	c.293-13A/C > G (IVS2-13 A/C- > G)	SW/SV
CAHAP12	USF	−	+	+	−	30 kb Del. (CH-7)[b]	30 kb Del. (Exon 7; p.Val281LeuL^p.Phe307fx)	SW
CAHAP13	USF	+	+	−	−	c.293-13A/C > G (IVS2-13 A/C- > G)	No identified mutation (c.738 + 41T > C)[c]	–
CAHAP14	USF	+	+	−	−	c.293-13A/C > G (IVS2-13 A/C- > G)	c.293-13A/C > G (IVS2-13 A/C- > G)	SW/SV

[a] Family relationships: NA14732—Mother, NA14733—Father, NA14734—Proband; DD1464—Mother, DD1463—Proband.
[b] Deletion breakpoints are based on nomenclature based from Chen et al. (2012).
[c] Novel intron 6 variation inherited on chromosome in trans with c.293-13A/C > G.

Δ8 nt^p.Ile172Asn; CH-1), two in intron 7 (p.Met239Lys^Leu307fx; CH-7), and two with apparent novel breakpoints within exon 7 (p.Val281LeuL^p.Phe307fx). The conversion allele contained an intragenic conversion track that includes CYP21A1P-like sequence from exon 1 to intron 2 sequence (p.Pro30Leu—c.293-13A/C > G). The remaining CAH mutations included the intron 2 splice site mutation c.293-13A/C > G (12/27), the exon 3 8 bp deletion (c.332-339del), (2/27), p.Ile172Asn (2/27), p.Val281Leu (2/27) and one previously described promoter micro-conversion allele (c.1-126C > T, c.1-110T > A) [33]. The one remaining chromosome without a known CAH causative allele contained a novel variation within intron 6, c.738 + 41T > C, of unknown function or effect. Bioinformatic analysis using the Human Splicing Finder tool did not indicate that the c.738 + 41T > C mutation created any putative cryptic mRNA splicing donor or acceptor sites. It is possible that a second mutation exists outside of the region amplified by this PCR method that may affect 21OH levels in the patient. There are documented cases of CAH patients with only one detected CYP21A2 allele and that the expression of disease may be related to a combination of unresolved multiple genetic and/or environmental factors [34–36].

4. Discussion

Molecular analysis of the CYP21A2 gene has been proposed as an alternate second-tier testing approach to reduce the high rate of false positive and during 17OHP NBS for CAH. In order for a molecular second-tier screen to be appropriate for CAH NBS, the method must accommodate the unique requirements of DBS. While the historic gold standard for detecting recombinant deletion and conversion alleles in CAH has been Southern blot analysis, it is impractical in a screening setting due to the quantities of genomic DNA required per sample as well as being too labor intensive. In order to begin developing a second-tier molecular screening assay for CAH, we have designed a locus-specific long-range PCR method that accurately and robustly amplifies the CYP21A2, CYP21A1P, and CYP21A2 30 kb deletion and large scale conversion alleles of CYP21A2. This method was validated using commercially available CAH-positive specimens, Florida families with probands affected with CAH, and normal population controls. The single long-range amplicon approach described here can reliably detect a variety of CAH gene rearrangements, and the CYP21A2 amplicon provides a sequencing template that can be used to detect causative mutations in the functional gene including the Exon 3 8 bp deletion allele. Furthermore, the method has been optimized to consistently and robustly amplify the CYP21A2 gene and recombination alleles from the patient-prepared DBS specimens.

The complexity of the chromosomal region containing CYP21A2 and the degree of similarity between this gene and the CYP21A1P pseudogene require a highly-specific amplification strategy to avoid false positive and false negative results. One important goal of this study was to characterize the overall variation of CYP21A2 and CYP21A1P from 60 normal controls to inform genotype analysis. Two recent studies have taken similar approaches in determining the sequence variability in both CYP21A2 and CYP21A1P, one in a German population [37] and the other in an ethnic Chinese population [38]. The results of those studies and the data presented here demonstrate that the comparison of the GenBank reference sequences for CYP21A2 and CYP21A1P does not adequately predict which sequence variants are specific to CYP21A2 or to CYP21A1P in the general population. The Tsai study points out that there is significant CYP21A2-like sequence in the pseudogene and that PCR assays designed to those sites to differentially amplify the functional CYP21A2 gene will lack specificity (P30-24%, V281-21%, Q318-10.8%, R356-33.8%). As with the Chinese population, the healthy controls from this study also had CYP21A2-like sequences in the pseudogene, but at different frequencies (P30-7%, V281-42%, Q318-21%, R356-62%), further supporting these findings.

The importance of determining what nucleotide positions are specific to either CYP21A2 or the pseudogene is underscored by the variation that was seen in the normal population controls at nucleotide positions c.*440C > T and c.*443T > C. Differences in the reference sequences for these sites in CYP21A2 (**C**GC**T**) and CYP21A1P (**T**GC**C**) have been utilized as the basis of locus-specific PCR amplification in multiple studies as well as determining the CYP21A2 copy number analysis by MLPA [6,17,21,23,37,39,40]. In the normal population controls characterized in this study, 15/60 individuals had aberrant PCR profiles for the CYP21A2, CYP21A1P, 30 kb deletion and large scale conversion amplifications when using locus specific primers to this site. These included 13 false positives for the conversion, 4 false positives for the 30 kb deletion and two false negatives for CYP21A2. Similarly, PCR amplification approaches designed to take advantage of the Exon 3 8 bp deletion (c.332_339del8) and Exon 6 mutation cluster c.710T > A, c.713T > A, c.719T > A (p.Ile236Asn, p.Val238Glu, and p.Met239Lys) for locus specificity also need to be interpreted with care. Two of the CAH probands genotyped in this study carried the Exon 3 8 bp deletion in the CYP21A2 gene. For these individuals, the initial PCR profile indicated that they were positive for the 30 kb deletion allele when the deletion site was used for locus specificity for the CYP21A2 gene. While the Day approach provides redundant coverage of Exon 3 by amplifying the promoter to Exon 6, the correct genotype can only be assigned after DNA sequencing. Thus, the dependency on the use of clinically relevant mutations for PCR specificity will lead to additional false positives and complicated genotypic analysis. Several methods for mutation analysis of CYP21A2, including allele specific PCR, primer extension and oligo ligation assays [17,18,20,23,38,41–44] are based upon either the Exon 10 3′ UTR region, or the Exons 3 and 6 mutations, raising the question of analytical specificity for these CYP21A2 mutation screening approaches. The single long-range PCR amplification method described in this study overcomes this lack of specificity in the 3′UTR region by exploiting the deletion region in the TNXA and TNXB genes downstream of CYP21A1P and CYP21A2 and also simplifies downstream analysis because it is not

dependent on what mutations may be present in the CYP21A2 gene. However, one consequence of extending the PCR amplification to the TNXA and TNXB genes is that this method requires that the RCCX (RP-C4-CYP21-TNX) module be present in the normal configuration of CYP21A1P-TNXA and CYP21A2-TNXB. Surveys of the RCCX region have demonstrated that when the module is present in more than two copies, trimodular or greater, the genes in a repeat are reordered and the uncommon CYP21A2-TNXA configuration does exist in the population [45,46]. While this configuration was not detected in this study, such chromosomes could result in a false-positive PCR result for the large scale conversion. This problem can be overcome by sequencing the gene conversion PCR product (Fig. 1A, primers CYP21A2-F and TNXA-R) if two mutations are detected that include an apparent gene conversion. This will determine if there is an actual gene conversion or the PCR product contains a normal CYP21A2 gene. With the increased specificity of this assay, this should be an infrequent occurrence since only one large gene conversion was detected in 40 chromosomes from CAH patients.

It should be noted that there are potential limits for molecular second tier screening for CAH. CYP21A2 mutation detection is specific for CAH that is due to 21OHD. A retrospective genotyping study of 61 CAH diagnosed samples from 810,000 newborns screened between January 1999 and December 2003 in Germany identified 59 patients with CYP21A2 mutations, one patient with 3β-hydroxysteroid deficiency and one patient with no CYP21A2 mutations [18]. In addition to considering CYP21A2 genotypes, this algorithm also automatically referred infants to an endocrinologist if 17-OHP levels in the DBS exceeded critical age and weight adjusted thresholds, which identified the infant with no CYP21A2 mutations and the infant with 3β-hydroxysteroid deficiency. Therefore, automatic referral for 17-OHP levels above critical thresholds would be needed to detect CAH due to causes other than mutations in CYP21A2. A second concern about using a molecular second-tier approach is that CAH is a critical disorder, and turn-around time for results is important to detect infants with CAH prior to a salt-wasting crisis. One advantage for a molecular assay, however, is that many states have implemented newborn screening for Severe-Combined Immunodeficiency, which requires a molecular assay for detection [47]. As a consequence, a time-saving approach could be achieved if a CAH molecular assay could utilize DNA extracts from the SCID screening or a repeat sample can easily be incorporated into high-throughput daily DNA extraction workflow followed by a CYP21A2 targeted-genotyping assay.

The long-range amplification approach described in this study specifically amplifies CYP21A2 without contamination from the CYP21A1P pseudogene and identifies the two common forms of CYP21A2 gene rearrangements. The DNA sequencing and copy number analysis results presented indicate that the method described here can reliably amplify CYP21A2 and the 30 kb deletion and conversion variants with locus specificity for both the forward and reverse PCR primer regions. This method also has the demonstrated specificity required for the development of a second-tier molecular assay with a low false-positive and false-negative rate that could serve as the basis for an appropriate assay for a NBS program. Our long-range amplification method has also been validated for the use with newborn screening samples in an ongoing collaboration of testing of CAH screen positive DBS specimens with a U.S. state newborn screening program. The specificity of the method is reflected in the low frequency of large gene conversions, 1.7% of all CYP21A2 mutation alleles (unpublished data), and this CYP21A2 amplification method can be utilized for either existing targeted CYP21A2 genotyping methods for the development of high-throughput genotyping assays that will be necessary for use in routine newborn screening.

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Conflict of interest statement

None of the authors have conflicts of interest relating to the contents of this manuscript. Furthermore, the findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control.

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